



Stuart, S. A., Hinchcliffe, J. K., & Robinson, E. (2019). Evidence that neuropsychological deficits following early life adversity may underlie vulnerability to depression. *Neuropsychopharmacology*, 44, 1623–1630. <https://doi.org/10.1038/s41386-019-0388-6>

Peer reviewed version

Link to published version (if available):  
[10.1038/s41386-019-0388-6](https://doi.org/10.1038/s41386-019-0388-6)

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# **Evidence that neuropsychological deficits following early life adversity may underlie vulnerability to depression**

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**Running title: Neuropsychological impairments in early life adversity**

## **Key words**

Affective biases, memory, reward, decision-making, corticosterone, antidepressant

**Abstract** (275 words)

**Article body** (4632 words)

**Figures** – 4

**Tables** – 1

**Supplementary Material** – supplementary methods, tables (3) and figures (8)

## Abstract

Early life adversity (ELA) is a risk factor for major depressive disorder (MDD), however the underlying mechanisms are not well understood. Clinical studies suggest that negative affective biases (the process whereby cognitive processes such as learning and memory and decision-making are modified by emotional state) represent a vulnerability factor for MDD. In this study we investigate the impact of ELA on affective biases and reward-associated behaviours in rats. Sprague Dawley rat pups underwent 14 days of postnatal maternal separation (180min/day from postnatal day 1: MS180) whilst control pups remained unhandled. In adulthood, affective biases associated with reward learning and decision-making were assessed using the affective bias test (ABT), or judgement bias task (JBT) respectively. Changes in motivation and reward sensitivity were tested in a progressive ratio (PR) schedule of operant responding and the sucrose preference test (SPT) respectively. We observed that MS180 animals expressed enhanced negative biases in response to acute corticosterone treatment but without effects on antidepressant-induced positive biases. ELA animals were impaired in their ability to develop appropriate biases in response to changes in reward value in a modified ABT but in the absence of any changes in reward sensitivity or motivation. No effects on decision-making were observed in the JBT but MS180 animals failed to develop the same more optimistic behavioural profile as controls in response to an increase in reward value. These findings suggest that ELA in rats increases vulnerability to negative affective biases and impairs animals' ability to appropriately learn reward value, independent of a reward sensitivity or changes in motivation. These data provide important evidence linking ELA with relevant neuropsychological impairments that may explain increased risk of developing MDD.

## Introduction

Major depressive disorder (MDD) is one of the most prevalent mental health conditions affecting modern society and it is projected to become the leading cause of global disability adjusted life years by 2030 (1). Our understanding of the aetiology of MDD is limited, and whilst epidemiological studies have implicated risk factors including early life adversity, chronic stress, and family history (2-4), how these relate to vulnerability are relatively unknown. In 1967, Beck first proposed that adverse experiences in early life contribute to the development of negative schemata that ultimately lead to negative biases in the processing of emotional information (5). More recently, studies in clinical populations suggest a key role of negative affective biases in learning, memory and decision-making in the development, maintenance and treatment of MDD and have led to a renewed interest in a cognitive neuropsychological hypothesis of depression and antidepressant action (6-8).

Negative affective biases across a number of cognitive domains have been observed in depressed patients, including negative interpretation of ambiguous information and a reduced memory for positively valenced stimuli (9-11). Not only do these biases tend to persist into clinical remission and have been linked to an increased risk of relapse, but they have also been observed in non-depressed individuals who are at high risk of developing MDD (12-15). This evidence indicates that, rather than being a simple marker of low mood, negative affective biases may represent a vulnerability factor for MDD.

The use of human neuropsychological tests to assay objective measures of affective biases in the clinic has provided us with the opportunity to use 'reverse translation' to evaluate similar neuropsychological processes in laboratory animals (for review see Hales *et al.* (16)). In particular we have developed the Affective Bias Test (ABT) to study biases in reward-learning and memory (17, 18). This task uses associative learning between specific cues (digging substrates) and reward (food pellet) to test the influence of affective state at the time of learning on the subsequent relative valuation of that reward-association. Validation experiments have shown that acute changes in

affective state at the time of learning biases subsequent choice behaviour in this assay (17). We have recently used a modified version of this task where animals are trained to associated one digging substrate with a higher value reward which, during the preference test, induces a bias towards that substrate, referred to as a *reward-induced positive bias*. We have shown that animals in putative depression-like states fail to develop this bias independent of effects on simple hedonic responses (19). The judgement bias task (JBT) is used to assay affective biases linked to decision-making behaviour, and test animal's interpretation of ambiguous information within the context of positive versus negative/less positive associations (20, 21). Together with other research groups we have shown that animals in putative negative affective states make more pessimistic choices in response to ambiguous cues (20-23).

Based on these initial findings we designed a series of studies to investigate the hypothesis that early life adversity (24, 25), is associated with negative affective biases in these tasks and this underlies vulnerability to MDD. Repeated maternal separation in rats is commonly used as a model of early life adversity, with animals demonstrating exaggerated responses to stress and depression-like behaviours in adulthood (26, 27). Our initial studies were designed to assess the impact of early life adversity on the development of stress- and reward-induced biases in the ABT, as well as interpretation biases in the JBT. We also assay consummatory anhedonia in the sucrose preference test (SPT), and reward motivation in an operant progressive ratio (PR) task, to compare the effects of early life adversity on distinct measures of reward processing.

## Methods and Materials

### Maternal Separation

The maternal separation procedure was adapted from Mirescu *et al* (28). Litters were bred in-house from 7 female and 2 male Sprague Dawley rats, and standardised to 10 pups (6-7 males, 3-4 females) on postnatal day (PND) 1. Litters were assigned to one of two rearing conditions from PND 1-14: handled with 180 mins maternal separation per day (MS180) or no maternal separation or handling (NS) (full details provided in Supplementary Material). On PND22, all rats were weaned and housed as same-sex and same-litter pairs. All experiments were carried out from PND 65 in male rats only. Three cohorts of adult male Sprague Dawley rats were used in these studies (outlined in Figure 1) with representation from different litters distributed across the cohorts. We also ran additional statistical analysis to check that there was no significant difference between litters within each rearing group (2-way ANOVA with REARING and LITTER as factors). Although the number of litters and therefore parental backgrounds were relatively small and are a limitation, our data suggest that the results were not influenced by litter effects. An example of the individual data for animals from the different litters is shown in the supplementary material (Figure S2). Validation of the MS180 model was achieved by measuring stress responsiveness (plasma corticosterone), neurogenesis (BrdU immunohistochemistry), novelty suppressed feeding and consummatory anhedonia (sucrose preference test).

Rats in cohort 1 weighed ~320-350g at the start of behavioural training for the ABT (PND65) and rats in cohort 2 weighed ~410-470g at the start of PR training (PND105). Rats in cohort 3 weighed ~330-350g at the time of BrdU injections (PND70). There was no significant difference in body weight at any of the time points post-weaning (Table S3). During behavioural testing rats were maintained at approximately 90% of their free-feeding weight by restricting access to laboratory chow (Purina, UK) to ~18g per rat per day. Water was provided *ad libitum*. All rats were maintained in temperature-controlled conditions on a 12:12h light-dark cycle (lights off at 07:00h), and behavioural testing was

carried out between 09:00-17:00 during the animals' active phase. All procedures received ethical approval by the UK Home Office and were conducted in adherence to the regulations of the 1986 Animals (Scientific Procedures) Act, EU Directive 2010/63/EU and ARRIVE guidelines (29).

### **Validation of the ELA model**

**Plasma corticosterone:** Blood samples were taken from the lateral tail vein immediately following removal of the animal from the home cage (baseline stress level), or on a separate occasion following 20 min in a restraint tube. Plasma corticosterone was determined by radioimmunoassay. Full methods are described in the Supplementary Material.

**Novelty suppressed feeding test (NSFT):** Rats were food deprived for 24 hours before being placed at the edge of a circular, opaque test arena (diameter: 70 cm, height: 50 cm) lined with sawdust and containing a ceramic bowl (diameter: 10cm) at the centre filled with standard laboratory chow. Latency to approach the food and latency to feed were recorded and the animal was returned to the home cage either once feeding commenced, or after a maximum of 15 min.

**BrdU immunohistochemistry and quantification:** Cell proliferation was assessed by staining for the exogenous thymidine analogue 5-bromodeoxyuridine (BrdU). Animals received 4 injections of BrdU (50mg/kg i.p.) at 2-hour intervals and were sacrificed 24 hours after the last injection. BrdU-positive cells in the hippocampus were visualised using a DAB-staining protocol (Supplementary Material). BrdU positive labelled cells were counted in the subgranular zone of the hippocampus from 6 bregma levels at 40-times magnification (-2.76, -3.24, -3.76, -5.40, -5.64, -6.00). Results were analysed as the total number of counted cells.

**Sucrose Preference Test (SPT):** Animals were acclimated to drinking a 1% sucrose solution from two drip-resistant water bottles for 48 hours (Ancare, USA). On the test day, animals were water restricted for 4 hours and moved into individual clean cages for 30 minutes before testing. The rats

were then given a 1 hour two-bottle choice sucrose consumption test (one bottle of water and one bottle containing a 1% sucrose solution).

### **Affective Bias Test (ABT)**

**General Protocol:** A detailed description of the training and testing procedure is provided in the Supplementary Material and in Stuart et al. (17). Animals were first trained to dig in bowl containing sawdust to retrieve a food reward (45mg rodent tablet, TestDiet, Sandown Scientific UK). Training was complete once each rat was able to find the pellets on 12 consecutive trials within 20 seconds for each trial. Animals then underwent a discrimination session consisting of discrete trials where the animal was placed into the test arena and allowed to approach and explore two bowls: one 'rewarded' substrate (CS+), and one 'blank' unrewarded substrate. (CS-). Once the animal started digging in one bowl, the other was removed by the experimenter. Animals were required to achieve 6 consecutive correct trials from a maximum of 20 trials to progress to testing.

**Drug-induced bias studies:** These studies followed a standard protocol of four pairing sessions followed by a preference test session on the fifth day. Each of the pairing sessions followed the same protocol as the discrimination session described above. A within-subject design was used wherein each animal learned to associate two different digging substrates (CS+A or CS+B) with a food pellet reward during pairing sessions. Independent pairing sessions (CS+A vs CS- or CS+B vs CS-) were carried out on days 1 - 4 (Figure S1A) and, on the fifth day, the rats were presented with both previously reinforced substrates together for the first time (CS+A vs CS+B) and their choices over 30 trials recorded. Drug-induced affective bias is established by pairing one session with pre-treatment of the test drug (either corticosterone or venlafaxine), and the other session with pre-treatment of vehicle. Treatment groups are outlined in Tables S1 and S2. We tested both corticosterone and



venlafaxine to determine whether animals showed an exaggerated response to negative biases specifically or whether ELA caused a more general increase in affective biases irrespective of the valence. Choice bias was calculated as the proportion of choices made for the drug-paired substrate vs. the total number of trials (drug-paired substrate + vehicle-paired substrate). A value of 50 was then subtracted from the choice bias score to give a % choice bias where a bias towards the drug-paired substrate gave a positive value and a bias towards the vehicle-paired substrate gave a negative value.

**Drugs:** Corticosterone was purchased from Sigma Aldrich, UK, dissolved in a 5% DMSO, 95% sesame oil vehicle, and administered at a dose range of 0-30 mg/kg via the subcutaneous (s.c.) route. Vehicle-treated animals received an s.c. injection of the DMSO/sesame oil vehicle. Venlafaxine was purchased from Hello Bio, UK, dissolved in 0.9% saline, and administered at a dose range of 0-30 mg/kg via the intraperitoneal (i.p.) route using a modified handling technique to minimise stress (Stuart and Robinson, 2015). Vehicle-treated animals in this study received 0.9% saline i.p. All doses were administered at a volume of 1ml/kg, 30 mins before ABT sessions. The dose ranges were selected based on previously published data using the ABT (17, 18).

**Reward-induced positive bias:** Each animal underwent pairing sessions as previously described, however one substrate (CS+A or CS+B) was paired with a single food pellet reward, and the other paired with 2 food pellets (Figure S1B) in the absence of any drug treatment. Preference test trials were run with a single pellet using the random reinforcement protocol.

### **Judgement Bias Task (JBT)**

**General protocol:** Rats were trained in an operant version of the JBT where they learned to make an active response (lever press) to an auditory tone predicting a positive 'P' outcome (receiving food

reward) and to make an active avoidance response (mount platform) to a light cue predicting a combined positive and negative 'P/N' outcome (receiving food reward and avoiding footshock). Previous studies have used cues which predict either obtaining reward or punishment avoidance which are thought to result in animals developing a positive valence and negative valence respectively (21, 30, 31). The avoidance response is time consuming to train and requires relatively high shock intensities risking development of learned helplessness, therefore, in this study we used a reward plus punishment avoidance association for the lower value association. Full details of the training and testing procedure are provided in the Supplementary Material. Briefly, training and testing sessions were carried out in six computer-controlled standard operant boxes (MedAssociates, Sandown Scientific, UK) fitted with a lever and custom-built insulated platform. K-Limbic software (Conclusive Solutions Ltd., UK) was used to program behavioural protocols and for data acquisition. Animals underwent a progressive training procedure involving initial training to the positive 'P' cue (2kHz, 75dB) where a lever press response resulted in delivery of a food reward (45mg rodent tablet, TestDiet, Sandown Scientific UK). Once animals reached 70% accuracy they progressed to positive and negative 'P/N'-cue training. For the P/N cue (yellow house light), an active response involved mounting the platform which avoided footshock and delivered a food pellet reward. Footshock was delivered at 0.1mA and increased by 0.01mA each day until animals achieved a criterion of 70% active avoidance. The final footshock amplitude was 0.2mA, and the maximum duration was 30s of 13 pulses [0.2s shock ON, 1.8s shock OFF] if the animals did not subsequently avoid the shock by mounting the platform. A summary of the stages used for training, number of sessions and trials at each stage are given in Table 1. There were no differences in training between the groups (RMANOVA with session and group as factors). The final stage of training involved a discrimination stage where rats were trained to discriminate between the P and P/N cues. Animals were considered trained once they achieved >70% accuracy over three consecutive days.

*Ambiguous (compound) cue testing:* Each testing session consisted of 40 trials each of the P and P/N cues, and 20 ambiguous (compound) cue trials. Each cue (20s presentation) was presented in randomised order and separated by a 10s ITI. A lever or platform response during compound cue trials resulted in random P or P/N outcomes. A lack of response during the 20s cue presentation was marked as an omission. Cognitive bias index (CBI) was calculated as the proportion of P/N responses to the ambiguous cue subtracted from the proportion of P responses, creating values in the range of -1 and 1. Negative values indicated a negative judgement bias and positive values indicated a positive judgement bias.

Animals were initially tested under the same contingencies as training however the results showed very negative biases in each group suggesting the relative saliency of the two outcomes were not appropriately balanced, resulting in a potential floor effect. To address this, animals were re-trained to new contingencies with the 'P' response paired with 2 food pellets and 'N' response paired with 1 food pellet and the avoidance of footshock. Animals were then re-tested using an ambiguous probe session.

### **Progressive ratio operant responding**

*General protocol:* Operant training was carried out in 8 identical computer-controlled operant chambers (dimensions 30.5 x 24.1 x 21.0 cm, MedAssociates, Sandown Scientific, UK) inside light- and sound-attenuating boxes. Only one lever (left or right, counterbalanced across all animals) was active during this experiment. The reinforcer was a single reward pellet (45mg rodent tablet, TestDiet, Sandown Scientific UK). Rats were initially trained on a fixed ratio (FR) 1 schedule then progressed to a gradual PR series: 1, 1, 2, 2, 3, 3, 4, 4, etc. The breakpoint for each animal was defined as the final completed ratio before responding ceased for a period of 5 min or more.

*Operant testing under a progressive ratio schedule:* Once animals reached a stable breakpoint (<10% change across 3 consecutive sessions), the motivational load of the task was increased for the testing sessions using the exponential PR schedule: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, .... This is derived from the formula  $[(5 \times e^{0.2n}) - 5]$ , rounded to the nearest integer, where  $n$  is the position in the sequence of ratios (32, 33). Animals were tested across 3 consecutive sessions under food restriction (high motivational state), and then given food *ad libitum* for 2 days before being tested in another 3 sessions under free feeding conditions (low motivational state).

### Statistical Analysis

All studies were performed with the experimenter blind to rearing group and drug treatment until the end of the study. All doses and other experimental factors were fully counter-balanced to avoid bias. A mixed-model ANOVA was used to analyse % choice bias data from dose-response experiments in the ABT (between-subject factor: GROUP, within-subject factor: DRUG) as well as % positive responses, response latency and % omissions in the JBT (between-subject factor: GROUP, within-subject factor: CUE). The % choice bias data from the reward-induced positive bias study, as well as CBI, sucrose preference, NSFT and plasma corticosterone data were analysed with an unpaired t-test comparing MS180 vs. NS group. *Post hoc* analysis for each treatment used a one-sample t-test against a theoretical mean of 0% choice bias where 0% is equivalent to 15 choices for the treatment- (drug or high reward) paired substrate and 15 choices for the control- (vehicle or low reward) paired substrate (17, 19). *Post hoc* analysis was made using pairwise comparisons between MS180 and NS groups if a significant main effect or interaction ( $p < 0.05$ ) was observed. Bonferroni correction was applied for multiple pairwise comparisons, Huynh-Feldt correction was used to adjust for violations of the sphericity assumption, and Levene's test was used to correct for inequality of variances. Statistical analyses were performed using SPSS Statistics 23 and graphs created using GraphPad Prism v7.

## Results

### Validation of the ELA model

*Plasma corticosterone:* There was no difference in plasma corticosterone levels at baseline. In the restraint stress experiment the MS180 had significantly higher corticosterone levels (unpaired t-test:  $t_9=6.16$ ,  $p=0.0002$ ,  $n=4-6/\text{group}$ ; three blood samples from the NS group and two from the MS180 group could not be obtained, and one data point from the NS group was an outlier [2SD from the mean] and so excluded; Figure 2A).

*NSFT:* MS180 animals had a longer latency to feed compared to NS animals in the NFST (unpaired t-test:  $t_{12}=3.06$ ,  $p=0.01$ ,  $n=8/\text{group}$ ; Figure 2B). There was no difference in approach latency between groups (Figure S3).

*Hippocampal BrdU:* MS180 animals showed a reduction in the number of total BrdU+ cells in the hippocampus compared to NS animals (unpaired t-test,  $t_5=3.17$ ,  $p=0.025$ ,  $n=4/\text{group}$ ; Figure 2C).

*SPT:* The MS180 and NS animals had a preference for a 1% sucrose solution over water, but there was no difference between groups (Figure 2D). There was no group difference in total fluid consumption (Figure S4).

### Affective Bias Test

Corticosterone induced a dose-dependent negative affective bias in both groups (RM ANOVA, DRUG:  $F_{2,28}=22.8$ ,  $p<0.001$ ; GROUP:  $F_{1,14}=10.7$ ,  $p=0.006$ ; DRUGxGROUP:  $F_{2,28}=1.66$ ,  $p=0.208$ ,  $n=8/\text{group}$ ; Figure 3A). However, at the lower dose of 10 mg/kg, MS180 rats showed a greater negative bias than the NS group (unpaired t-test:  $t_{14}=2.95$ ,  $p=0.01$ ). Venlafaxine induced a dose-dependent positive affective bias with no group difference (DRUG:  $F_{2,28}=16.6$ ,  $p<0.0001$ , GROUP:  $F_{1,14}=0.21$ ,

$p=0.66$ , DRUG $\times$ GROUP:  $F_{2,28}=0.08$ ,  $p=0.923$ ,  $n=8/\text{group}$ ; Figure 3B). In the modified version of the ABT, NS rats developed a reward-induced positive bias for the higher value substrate, an effect that was not observed in the MS180 group (unpaired t-test:  $t_{14}=5.4$ ,  $p<0.0001$ ,  $n=8/\text{group}$ ; Figure 3C).

### **Judgement bias task (JBT)**

There was no difference in CBI between the MS180 and NS animals in the initial JBT with both groups exhibiting a negative CBI (one sample t-test vs. CBI = 0.0: [NS:  $t_7=9.19$ ,  $p<0.0001$ ], [MS180:  $t_6=7.07$ ,  $p=0.004$ ],  $n=8/\text{group}$ ; Figure 3D). There was a main effect of CUE for the percentage of positive lever presses (RM ANOVA,  $F_{2,26}=629$ ,  $p<0.0001$ ), showing the animals were able to discriminate between previously learned cues, however there was no effect of GROUP or GROUP  $\times$  CUE interaction (Figure S5). There were no main effects of ELA on response latency or omissions (Figure S6). After the relative value of the outcomes was altered, the MS animals failed to shift their bias in the same, more positive direction as the controls suggesting a failure to integrate the new reward information resulting in a relatively higher level of anticipation of negative events compared to controls. A significant effect on CBI was observed, with the NS animals showing a less negative score compared to the MS180 group (unpaired t-test,  $t_{14}=2.23$ ,  $p=0.043$ ; Figure 3D). There was a main effect of GROUP (RM ANOVA,  $F_{1,14}=5.95$ ,  $p=0.029$ ) and GROUP  $\times$  CUE interaction (RM ANOVA,  $F_{2,28}=3.46$ ,  $p=0.046$ ; Figure S7) on the percentage of positive lever presses. *Post-hoc* analysis revealed that NS animals made a greater percentage of responses on the positive lever in response to the ambiguous, compound cue compared to MS180 animals (post hoc pairwise comparisons,  $p=0.043$ ; Figure S7). The NS group also showed a trend towards greater responding on the positive lever in response to the N cue (post hoc pairwise comparison,  $p=0.066$ ; Figure S6). There was no effect of group on response latencies or omissions (Figure S8).

### **Progressive ratio operant responding**

There was no effect of ELA on the break point of responding across the 10 training sessions analysed (Figure 4A). There was also no observed difference between MS180 and NS animals under conditions of food restriction or free food (Figure 4B).

## Discussion

In the present study we show that repeated maternal separation of rat pups induces behavioural and physiological changes in adulthood that are characteristic of an animal model of early life adversity. MS180 animals demonstrate an exaggerated corticosterone response to acute restraint stress, as well as increased hyponeophagia compared to control animals. The number of litters we have sampled from may present a limitation to the behavioural data, however these effects have been well described in the literature and have also been seen in other animal models of depression, including chronic mild stress (27, 34, 35). We also show that the model causes a decrease in hippocampal neurogenesis consistent with previous studies in rats (28). These findings suggest that our MS180 method induced the expected phenotype and confirmed the presence of a deficit in adult animals.

We then carried out a series of studies that have revealed some important characteristics of the model in relation to affective biases. We have shown that animals subjected to ELA appear to have an increased sensitivity to corticosterone-induced negative bias in adulthood. The dose-response curve is shifted such that these animals show a negative affective bias at a dose of the drug which does not induce a negative bias in control animals. This effect may relate to the exaggerated responses to stressors characteristic of ELA models and links this vulnerability to an increased propensity to negative affective biases. Using the modified version of the ABT, we are able to investigate the effects of ELA on reward-related learning and memory and subsequent anticipation of reward. In this assay the animals must make a decision about which substrate-reward cue to

select based on their prior experience of the associated reward value. We show that normal rats develop a positive bias towards the substrate that has previously been paired with a higher value of reward. This is consistent with studies showing that rodents will learn to associate a cue with a higher value reward and subsequently demonstrate a preference for that cue over one that predicts a lower value reward (36-38). This reward-induced positive bias is lost in the ELA model which we suggest reflects a failure to appropriately anticipate the greater value of the substrate paired with the higher value reward. We have previously shown that animals treated chronically with 'pro-depressant' drugs, ie. drugs that are known to increase the risk of negative affective states such as MDD in humans, results in a similar deficit in reward-induced positive bias (19). Interestingly our data show that this effect occurs in the absence of a reduced preference for a 1% sucrose solution vs. water, replicating findings from other groups that fail to show an effect of ELA in the SPT (26, 39). Together with our current data, these findings would suggest that the reward deficits observed in the ABT are independent of the hedonic response as measured by the SPT. While tasks like the SPT are able to quantify consummatory aspects of reward, and how animals experience pleasure at the time of consumption, the deficits in human depression are more complex (40, 41). Depression is more commonly associated with impairments in anticipation of reward which we propose represent an interaction between cues which predict reward, activation of memory processes, and subsequent recall of expected reward value, which then drive goal directed behaviour. Whilst various methods involving chronic stress have been shown to decrease sucrose preference in rodents (42), several researchers have been unable to replicate these findings (43-45). These results from human and animal studies suggest that perhaps the SPT is less suitable as an assessment of anhedonia relevant to depressive disorders, and we propose that our current work adds to previous observations that reward deficits measured using the modified ABT may be more relevant to the reward deficits seen in MDD.

Previous studies using the JBT have shown that chronic stressors induce enduring negative biases in decision-making (22, 23). In the first study, no differences were observed between groups however,



the groups both showed very negative CBIs which may have resulted in a floor effect. As this was a new version of the task involving reward plus punishment avoidance, we cannot be sure whether the relative valence and value was learnt in line with our predictions. After the relative value of the outcomes was altered, the MS animals failed to shift their bias in the same, more positive direction as the controls suggesting a failure to integrate the new reward information resulting in a relatively higher level of anticipation of negative events compared to controls. As the animals were re-trained to new contingencies, the effect may have been related to a failure of the MS animals to update cue-reward associations in order to drive appropriate decision-making behaviour. When the value of the cue predicting a positive outcome is increased, normal animals modify their behaviour and show a less pessimistic interpretation of an ambiguous cue. The failure of animals that have undergone ELA to similarly shift their responses in this task could be due to a failure to integrate new information about the value of a reward-associated cue into their decisions.

It is important to note that the pessimistic judgement biases exhibited by both treatment groups is characteristic of reward vs. punishment versions of the JBT (20). In a more recent version of the task where the two learned cue-outcome associations are linked to different values of reward (high reward vs low reward), rats instead show a positive judgement bias that becomes more negative after chronic restraint stress (21). This version may therefore be useful in further investigating the effects of ELA on judgment biases in rats.

### **Clinical Implications**

Together our findings support the evidence from clinical studies that stress in early life leads to lasting changes in neuropsychological processes relevant to MDD. ELA represents a strong vulnerability factor for MDD and our work suggests that it causes increased sensitivity to acute stress-induced negative affective biases in learning, memory and pessimistic decision-making. These may play a key role in the development of MDD in adulthood. Indeed prospective studies in patients

have shown that negative processing biases, for example when interpreting ambiguous stimuli, predict negative affect in response to stress (46).

Our work has also revealed key differences between reward-related learning and memory deficits in the ABT and behaviours measured using the SPT and PR tasks, suggesting a distinction between hedonic and motivational deficits compared with cognitive aspects of reward processing. The deficits observed in the ABT and the ELA model compare favourably with findings in MDD patients who demonstrate a deficit in goal-directed behaviours linked to the anticipation of reward in the absence of consummatory anhedonia (40, 41). Further studies to test whether antidepressant treatment is able to remediate the deficits in reward processing present in ELA animals is warranted to determine a causal relationship of negative affective biases in MDD. Overall our findings support a neuropsychological hypothesis in the vulnerability to MDD and why at-risk individuals who have previously experienced ELA may be more likely to exhibit changes in reward processing and develop negative biases during periods of stress.

## **Funding and Disclosures**

This research was funded by the Medical Research Council (MR/L011212/1). JKH is funded by a University of Bristol PhD Studentship. The authors have no biomedical financial interests or potential conflicts of interest relating to this work. ESJR has current or previous research grant funding from Eli Lilly, Pfizer, Boehringer Ingelheim and MSD but these companies have not been directly involved with this research or this manuscript.

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## Figure Legends

### Figure 1. Outline of the study protocol.

Three cohorts of rats acquired from the maternal separation protocol were used for the behavioural and immunohistological studies. The above schematic outlines the time points of each experimental procedure. PND: postnatal day, MS180: Maternal separation 180 min/day procedure, NS: non-separated control animals, ABT: affective bias test, SPT: sucrose preference test, CORT: corticosterone, JBT: judgement bias task, PR: progressive ratio, NFST: novelty suppressed feeding test.

### Figure 2. Validation of the ELA model.

Maternal separation (MS180) increases the corticosterone response to acute restraint stress (panel A), increases the feeding latency in a novel environment (panel B), and reduces the total number of BrdU-positive cells in the hippocampus (panel C) compared to non-separated (NS) animals. MS180 animals do not show reduced sucrose preference in the SPT (panel D). Data represents the mean  $\pm$  SEM, # $p < 0.05$ , ### $p < 0.001$ ;  $n = 4-6$ /group (CORT response),  $n = 8$ /group (NSFT),  $n = 4$ /group (BrdU),  $n = 8$ /group (SPT).

### Figure 3. ELA increases corticosterone-induced negative affective bias and impairs reward-induced positive bias in the ABT and animals show impaired responses to an increase in reward in the JBT.

Acute treatment with corticosterone induces a negative affective bias in rats in the ABT, with maternal separation (MS180) animals showing a greater bias at a lower dose compared to non-separated (NS) animals (panel A), however there was no effect of ELA on positive bias induced by the antidepressant, venlafaxine (panel B). MS180 animals show a significant deficit in reward-associated

positive bias (panel C). Both MS180 and NS groups show a negative cognitive bias index (CBI) when the positive (P) cue predicts a low value of reward in the JBT (panel D). When the reward value is increased, NS but not MS180 animals shift towards a less negative CBI, indicating a less pessimistic judgement bias. Data represent the mean  $\pm$  SEM, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs 0% choice bias, # $p < 0.05$ , ### $p < 0.001$  vs NS;  $n = 16/\text{group}$  (ABT),  $n = 7$  per group (low reward JBT),  $n = 8$  per group (high reward JBT).

**Figure 4. ELA has no effect on motivation to obtain reward in an operant progressive ratio task.**

Maternal separation (MS180) animals show no difference from non-separated (NS) controls in performance during training on a progressive ratio schedule (gradual PR series: 1, 1, 2, 2, 3, 3, 4, 4, etc) of reward (left panel). Using the more challenging test schedule (1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50 etc), no effect was observed during either a state of high motivation (food restriction), or low motivation (free food) (right panel). Data shown as mean  $\pm$  SEM,  $n = 8/\text{group}$ .

## Tables

**Table 1.** Summary of JBT training procedure.

Stage	Details	Number of sessions to criteria	Number of trials per session	Group difference
1	Reward training	16	100 trials	none
2	Punishment training stage 1	5	50 trials	none
3	Punishment training stage 2	6	30 trials	none
4	'P' vs 'P' discrimination, Lever - 1 pellet vs platform - 1 pellet	8	100 trials	none
5	'P' vs 'P/N' discrimination, Lever - 1 pellet vs platform - 1 pellet and footshock avoidance	12	100 trials	none
6	'P' vs 'P/N' discrimination, Lever - 2 pellets vs platform - 1 pellet and footshock avoidance	3	100 trials	none



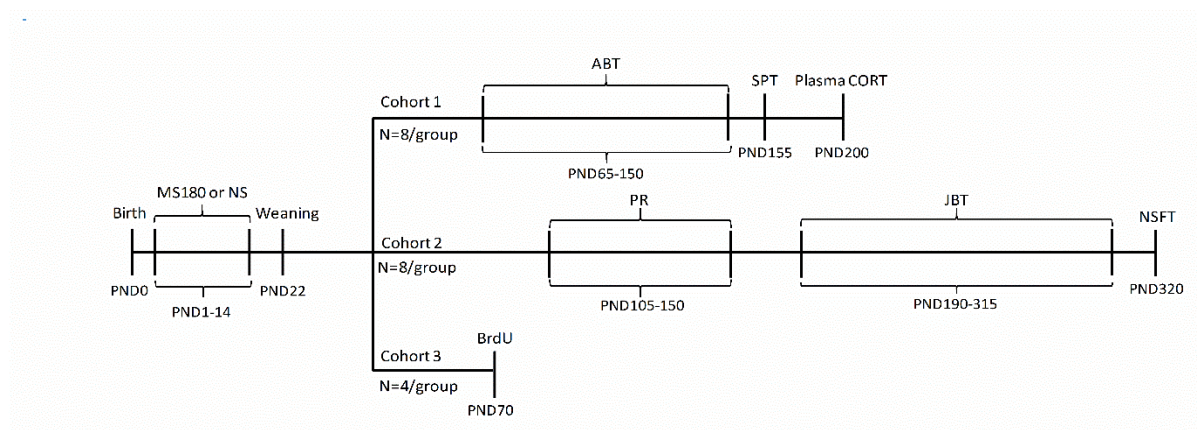
**Fig 1**

Fig 2

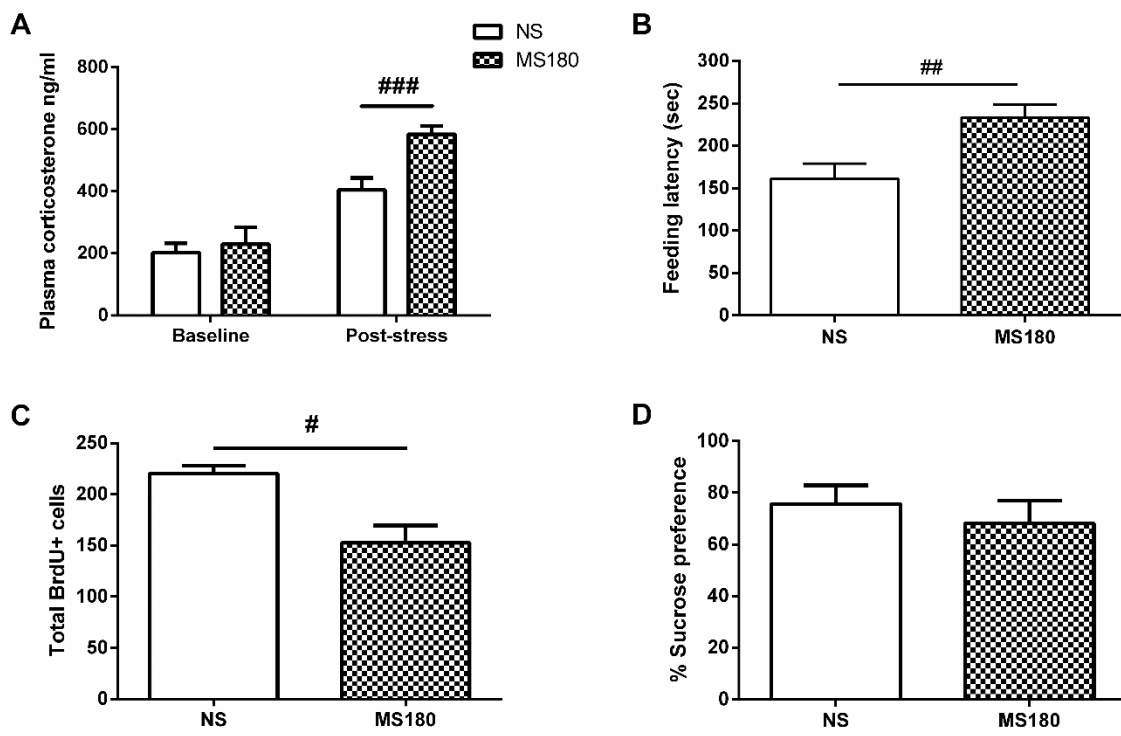


Fig 3

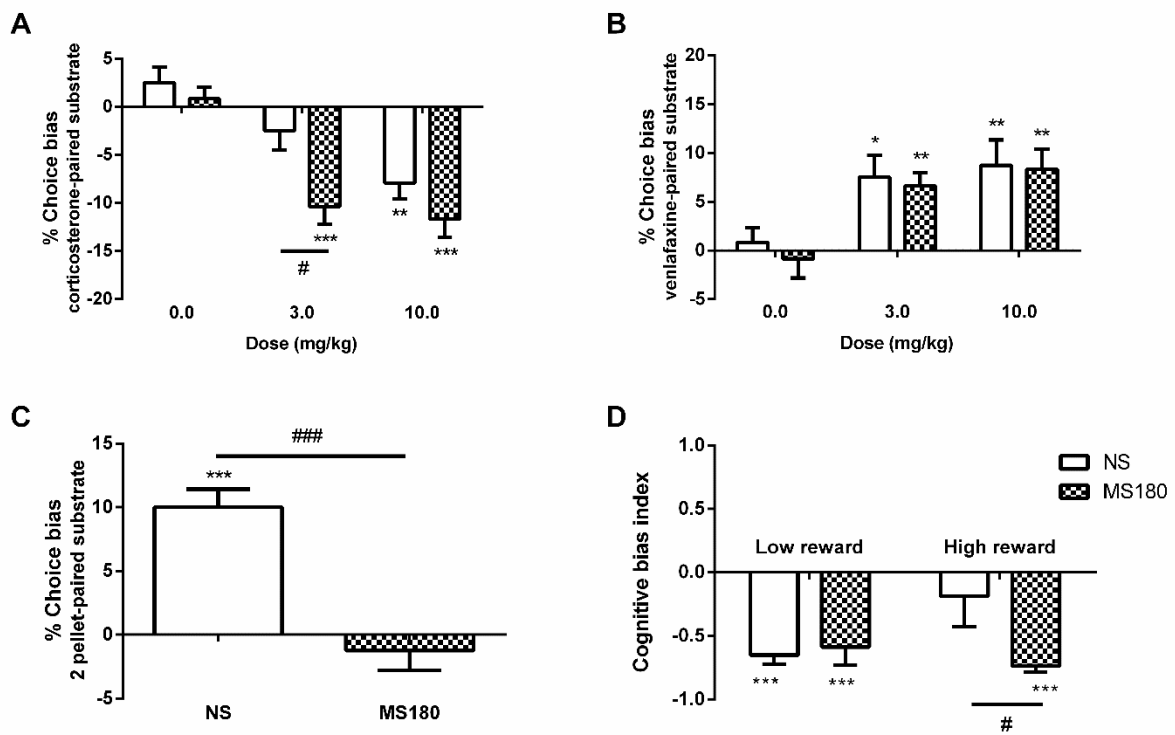
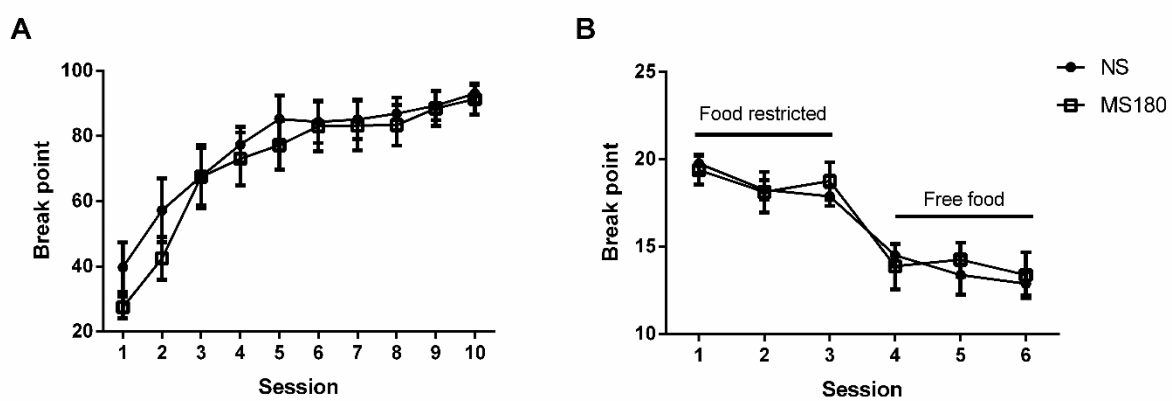


Fig 4



## Supplementary Material

### METHODS

#### Maternal separation

The maternal separation procedure was adapted from Mirescu *et al* (28). Litters were bred in-house from 7 female and 2 male Sprague Dawley rats, and standardised to 10 pups (6-7 males, 3-4 females) on postnatal day (PND) 1. Litters were assigned to one of two rearing conditions from PND 1-14: handled with 180 mins maternal separation (MS180) or no handling and no separation (NS). Regular cage cleaning ceased during this period to avoid disturbing home cage nests. Rats were provided with *ad libitum* access to food and water. For each of the MS180 litters, pups were transferred to a plastic container lined with bedding from the home cage and placed in an incubator maintained at 31-33 °C for 180 mins between 10:00-13:00h. After the separation period, pups were returned to the home cage. NS litters were left undisturbed until PND14, at which time normal cage cleaning resumed. On PND22, all rats were weaned and housed as same-sex and same-litter pairs. All experiments were carried out from PND 65 in male rats.

#### Blood sampling

Blood samples (~100ul) were taken from the lateral tail vein, either immediately following removal from the home cage (baseline stress level), or following 20 min of restraint in a 8cm diameter plastic tube. Samples were collected in Eppendorf tubes containing 0.5M EDTA and centrifuged at 4000rpm for 10 mins to pellet cells, with plasma supernatant removed and stored at -20°C until analysis.

### **Plasma corticosterone radioimmunoassay**

A corticosterone standard curve was created using 1:2 serial dilutions of a 100ng/ml of corticosterone (Sigma-Aldrich, UK) in citrate buffer in addition to two quality controls (QCs) of 100ng/ml and 20ng/ml corticosterone. Each 10ul plasma sample was diluted to 500ul in citrate buffer (25mM tri-sodium citrate, 50mM sodium dihydrogen orthophosphate, 1g/l BSA (Sigma-Aldrich, UK)) to denature corticosterone-binding globulins. All standards, QCs and samples were processed in 100ul triplicate aliquots and were incubated at 4°C overnight containing 50ul of primary antibody (20mg antibody dissolved in 0.2ml dH<sub>2</sub>O, 9.8ml 0.9% saline, 500ml citrate buffer) and tracer. All samples were then mixed with a dextran/charcoal T70 solution (0.05g Dextran T70 (Pharmacia Biotech, Sweden) and 0.5g activated charcoal (Sigma-Aldrich, UK)) and centrifuged for 15 minutes at 4000rpm (4°C). The supernatant for each sample was then aspirated and loaded onto a gamma counter (E5010 Cobra II Auto Gamma, Perkin Elmer, Netherlands). The specific activity of the tracer was 10uCi/ml. The assay had a limit of detection of 1ng/ml. The intra- and interassay coefficients of variation of the corticosterone assay were 16.7 and 13.3%, respectively. Primary rabbit anti-rat corticosterone antibody (supplied by Gábor Makara, Institute of Experimental Medicine, Bupapest, Hungary) and [125I]-corticosterone tracer (Institute of Isotopes, Hungary) were provided by Dr Becky Conway-Campbell and Professor Stafford Lightman (School of Clinical Sciences, University of Bristol).

### **BrdU immunohistochemistry and quantification**

Cell proliferation was assessed by staining for the exogenous thymidine analogue 5-bromodeoxyuridine (BrdU), which is incorporated into the DNA during the S phase of the cell cycle. Animals from cohort 3 (n=4/treatment group) received 4 injections of 50mg/kg BrdU at 2-hour intervals. Twenty-four hours after the last injection animals were sacrificed by transcardial perfusion with 0.01M PBS, followed by 4% paraformaldehyde (PFA). Brains were removed and post-fixed

overnight in PFA and subsequently cryoprotected in a 30% sucrose solution. Coronal sections (40µm,) were cut on a freezing microtome and stored in cryoprotectant until further processing.

Free-floating brain sections were washed in 0.01M PBS + Triton X-100 (PBS-T) to remove cryoprotectant and then incubated for 30 min in 2M HCl at 37 °C. The acid was neutralised 0.1M sodium tetraborate (pH 8.5) and sections were washed again in PBS-T. Sections were then incubated in 3% H<sub>2</sub>O<sub>2</sub> for 10 min to block endogenous peroxidase, washed with PBS-T and preincubated for 30 min with PBS-T, 3% horse normal serum and 2% bovine serum albumin (blocking solution). Sections were then incubated under agitation overnight at room temperature in primary mouse monoclonal anti-BrdU antibody (1:500) in PBS-T and 3% horse normal solution. Sections were washed in PBS-T and then incubated for 90 mins in biotinylated horse-anti-mouse antibody (1:1000). After further washing in PBS-T, sections were incubated in streptavidin–horseradish peroxidase (ExtrAvidin Peroxidase, 1:1000 in PBS) for 90 mins at room temperature, rinsed, and reacted with 3,3'-diaminobenzidine) DAB/nickel solution. After staining sections were mounted onto glass slides and dehydrated using 10 min washes of 95% ethanol, 100% ethanol (x3) and xylene (x2). Slides were then coverslipped using DPX mounting medium.

BrdU positive labelled cells were counted in the subgranular zone of the hippocampus from 6 bregma levels at 40-times magnification (-2.76, -3.24, -3.76, -5.40, -5.64, -6.00). Results were analysed as the total number of counted cells.

### **Affective bias test**

General Protocol: The rats were habituated to a 40x40cm Perspex test arena and trained to dig in two ceramic bowls (10cm diameter) filled with sawdust to obtain food pellets (45mg rodent tablet, TestDiet, Sandown Scientific UK). Digging training was complete once each rat was able to find the pellets on 12 consecutive trials within 20 seconds for each trial. Once trained to dig in sawdust,

animals underwent a discrimination session consisting of discrete trials where the animal was placed into the test arena and allowed to approach and explore two bowls: one 'rewarded' substrate (CS+), and one 'blank' unrewarded substrate. (CS-). Once the animal started digging in one bowl, the other was removed by the experimenter, the latency to dig recorded and the trial recorded as correct (rewarded substrate) or incorrect (blank substrate). If the animal failed to approach the bowls and dig within 20 seconds, the trial was recorded as an omission. Animals were run until they completed 6 consecutive correct trials, within a maximum of 20 trials.

*Drug-induced bias studies:* These studies were carried out as outlined in Figure S1A. Each animal learned to associate two different digging substrates (CS+A or CS+B) with obtaining a food pellet reward during pairing sessions. Independent pairing sessions (CS+A vs. CS- or CS+B vs. CS-) were carried out on days 1 - 4 , and on day 5 the rats were presented with both previously reinforced substrates together for the first time (CS+A vs. CS+B), and their choices over 30 trials recorded. For the preference test trials, a single pellet was placed in one of the bowls using a random reinforcement protocol such that there was a 1 in 3 probability for each substrate. Drug induced affective bias is established by pairing one session with pre-treatment of the test drug (either corticosterone or venlafaxine), and the other session with pre-treatment of vehicle. The substrate, pairing session and treatments (ie. drug or vehicle) were fully counter-balanced for each week of the study (Table S1). The drug doses were administered according to a fully randomised Latin square design with all animals receiving all doses by the end of the study (Table S2). In the reward-induced positive bias version of the assay, one substrate was paired with a high reward, and the other with a low reward, in the absence of drug (Figure S1B).



Figure S1. The Affective bias test.

**A. Drug-induced affective bias**

Day 1	Day 2	Day 3	Day 4	Day 5
Substrate A reward pairing CS+A vs. CS-	Substrate B reward pairing CS+B vs. CS-	Substrate A reward pairing CS+A vs. CS-	Substrate B reward pairing CS+B vs. CS-	Preference Test (30 trials) CS+A vs. CS+B
Drug (Corticosterone OR Venlafaxine)	Vehicle	Drug (Corticosterone OR Venlafaxine)	Vehicle	

**B. Reward-induced positive bias**

Day 1	Day 2	Day 3	Day 4	Day 5
Substrate A reward pairing CS+A vs. CS-	Substrate B reward pairing CS+B vs. CS-	Substrate A reward pairing CS+A vs. CS-	Substrate B reward pairing CS+B vs. CS-	Preference Test (30 trials) CS+A vs. CS+B
High reward	Low reward	High reward	Low reward	

**Table S1:** Standard procedure for testing drug-induced affective bias versus vehicle. Each animal receives drug treatment or vehicle counterbalanced over the four substrate-reward pairing sessions. Substrate and day are also counter-balanced resulting in four different groups.

	Day 1	Day 2	Day 3	Day 4	Day 5
	Pairing 1	Pairing 2	Pairing 3	Pairing 4	Preference Test
<b>Group 1</b>	CS+A vs. CS- Drug	CS+B vs. CS- Vehicle	CS+A vs. CS- Drug	CS+B vs. CS- Vehicle	CS+A vs. CS+B, 30 trials
<b>Group 2</b>	CS+B vs. CS- Drug	CS+A vs. CS- Vehicle	CS+B vs. CS- Drug	CS+A vs. CS- Vehicle	CS+A vs. CS+B, 30 trials
<b>Group 3</b>	CS+A vs. CS- Vehicle	CS+B vs. CS- Drug	CS+A vs. CS- Vehicle	CS+B vs. CS- Drug	CS+A vs. CS+B, 30 trials
<b>Group 4</b>	CS+B vs. CS- Vehicle	CS+A vs. CS- Drug	CS+B vs. CS- Vehicle	CS+A vs. CS- Drug	CS+A vs. CS+B, 30 trials

**Table S2: Corticosterone and venlafaxine-induced affective biases**

Group A = NS, Group B = MS180

Four way counter-balanced design using protocol 1 (table S2) where 'Drug' was:

	Corticosterone (mg/kg)			
	Week 1	Week 2	Week 3	Week 4
<b>Group 1</b>	0	3	10	30
<b>Group 2</b>	3	30	0	10
<b>Group 3</b>	10	0	30	3
<b>Group 4</b>	30	10	3	0

	Venlafaxine (mg/kg)			
	Week 1	Week 2	Week 3	Week 4
<b>Group 1</b>	0	3	10	30
<b>Group 2</b>	3	30	0	10
<b>Group 3</b>	10	0	30	3
<b>Group 4</b>	30	10	3	0

### Judgement Bias Task (JBT)

*Apparatus:* Training and testing sessions were carried out in six computer-controlled standard operant boxes (MedAssociates, Sandown Scientific, UK). Each chamber (ENV-008CT, dimensions 30.5 x 24.1 x 21.0 cm) located inside a light- and sound-attenuating box (ENV-018MD), was equipped with an audio generator (ANL-926), a pellet magazine and a pellet dispenser (ENV-203M-45), house and magazine light (28 V, 100 mA), two retractable response levers, a Perspex platform connected to lever (4x4cm), and a grid floor through which electric foot shock (0-1.0 mA) could be delivered (ENV-005). The lever and the platform were located on the rear wall of the box, on the opposite side to the magazine. K-Limbic software (Conclusive Solutions Ltd., UK) was used to program behavioural protocols, to control operant chambers, audio and shock generators, and for data acquisition.

*Positive 'P'-cue Training:* The first training stage involved tone presentations (2kHz, 75dB, 5sec) co-occurring with delivery of a single food pellet, separated by 10s inter-trial intervals (ITIs). Animals were then trained to press the left lever in response to the P cue (2kHz, 75dB, 20s) to receive a food

pellet, separated by a 10s ITI. A lever press during the ITI resulted in 5s timeout. Lever training continued until animals accomplished stable performance (over 70% correct). All P-cue training sessions consisted of 100 trials or lasted a maximum of 60 mins.

*Positive and negative 'P/N'-cue Training:* Rats were trained to mount an insulated platform located on the far left of the box, placed under the lever in response to P/N cue presentation to obtain a single food pellet and to avoid a footshock delivered through the grid floor. When animals mounted the platform, they could escape footshock and concurrently depress the lever to obtain a reward. The P/N training involved the presentation of the yellow house light (20s) with simultaneous delivery of footshock unless the animal pressed the platform, terminating the shock and cue presentation, followed by a 30s ITI. Footshock was delivered at 0.1mA and increased by 0.01mA each day until animals achieved a criterion of 70% active avoidance.

The next stage involved punishment training with a delayed onset of shock application. At this stage if the animal pressed the platform during the cue presentation (30s), the footshock was avoided and instead a 10s ITI was initiated and the trial recorded as a 'correct' response. If the animal failed to press the platform during the cue presentation, the footshock was delivered. A platform-press once the footshock had commenced, resulted in its termination and the trial was recorded as an 'escape'. Each platform-press during the ITI resulted in 5s timeout.

The final training stage involved learning the platform-cue association with the combined P/N outcome, where a platform-press during the cue presentation resulted in a single food pellet delivery and shock avoidance. A lack of response when the P/N cue was displayed resulted in shock delivery (30s of 13 pulses [0.2s shock ON, 1.8s shock OFF]) and no food pellet was dispensed. All 'P/N'-cue training sessions lasted a maximum of 30min or consisted of 50 (stage 1) or 30 (stage 2) trials. Animals were required to reach a minimum of 70% correct responses at each stage in order to progress to the next training stage.

*Discrimination training:* During this stage, the rats were trained to accurately discriminate between the P and P/N cues. Discrimination sessions consisted of 50 presentations of each cue (20s) in random order with a 10s ITI. A failure to make the appropriate response (ie lever press for the P cue, platform press for the P/N cue) was recorded as an incorrect response. Omissions, escapes and incorrect responses were scored separately. All discrimination training sessions consisted of 100 trials or lasted a maximum 60 mins. Animals were trained until they achieved 70% of correct responses with lever and platform, and continuing stable performance over three consecutive days, before proceeding to ambiguous cue testing.

*Ambiguous (compound) cue testing:* Each testing session consisted of 40 trials each of the P and P/N cues, and 20 ambiguous (compound) cue trials. Each cue (20s presentation) was presented in randomised order and separated by a 10s ITI. A lever or platform response on compound cue trials resulted in random P or P/N outcomes to prevent new cue-outcome associations forming during testing. A lack of response during the 20s cue presentation was marked as an omission. All responses: correct (i.e. accurate lever or platform press to the corresponding cue), incorrect (i.e. wrong lever or platform press to the tone or the light cue) and during compound cue presentation were scored and converted into percentage of the total number of responses to an applied cue. Cognitive bias index (CBI) was calculated as the proportion of P/N responses to the ambiguous cue subtracted from the proportion of P responses, creating values in the range of -1 and 1. Negative values indicated a negative judgement bias and positive values indicated a positive judgement bias.

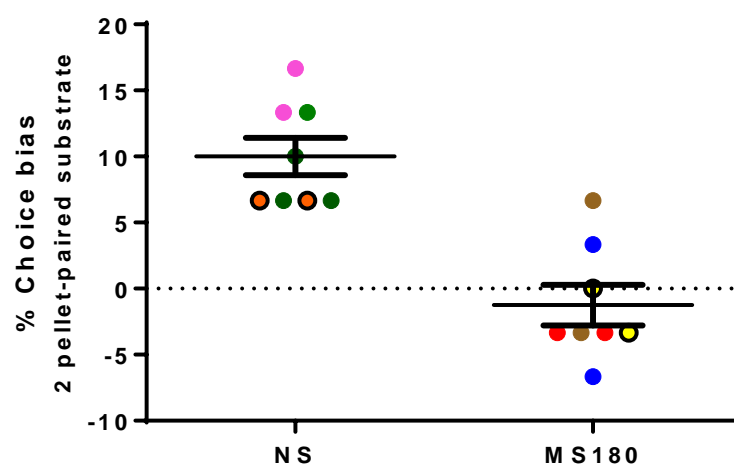
Animals were initially tested under the same contingencies as training however the results showed very negative biases in each group suggesting the relative saliency of the two outcomes were not appropriately balanced, resulting in a potential floor effect. To address this, animals were re-trained to new contingencies with the 'P' response paired with 2 food pellets and 'N' response paired with 1 food pellet and the avoidance of footshock. Animals were then re-tested using an ambiguous probe session.

## RESULTS

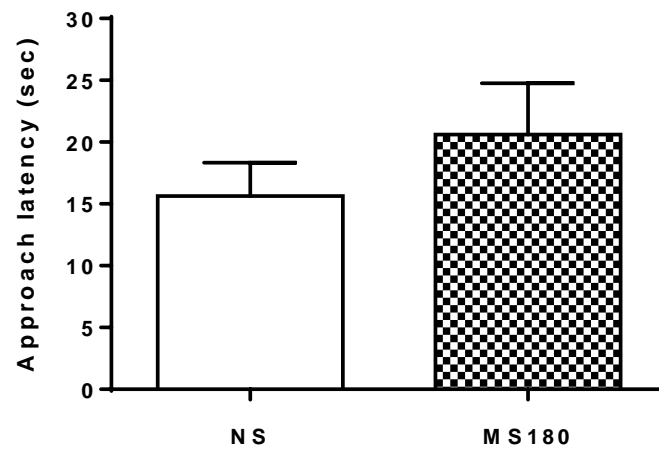
**Table S3.** Body weight was unaffected by maternal separation (MS180) in post-weaning rats when compared to non-separated animals (NS). Data represents the mean  $\pm$  SEM.

Animal	N	PND 21	PND 70	PND 100	PND 180	PND 320
<b>Non-Separated (NS)</b>	<b>20</b>	56.6 $\pm$ 0.7				
Cohort 1	8		334.8 $\pm$ 4.2	433.1 $\pm$ 4.7		
Cohort 2	8			485.1 $\pm$ 10.4	571.0 $\pm$ 16.4	634.6 $\pm$ 14.7
Cohort 3	4		343.5 $\pm$ 8.3			
<b>Maternally Separated (MS180)</b>	<b>24</b>	58.2 $\pm$ 0.9				
Cohort 1	8		328.8 $\pm$ 8.2	428.9 $\pm$ 15.7		
Cohort 2	8			463.1 $\pm$ 10.6	540.8 $\pm$ 17.6	602.8 $\pm$ 17.9
Cohort 3	4		327.5 $\pm$ 4.6			

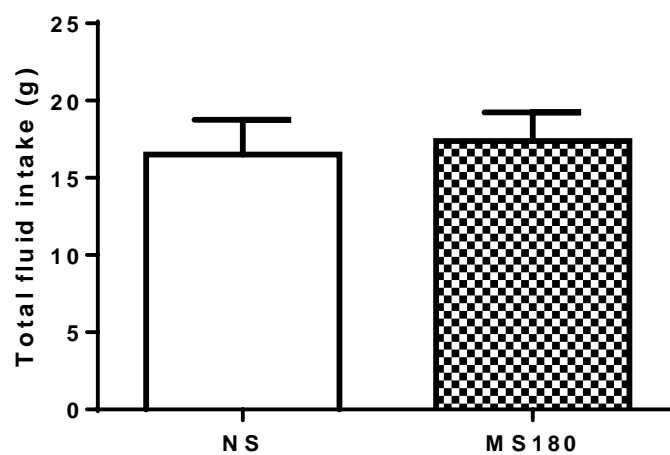
**Figure S2.** There were no between-litter effects on reward-induced positive bias in the ABT. Data represents the mean bias  $\pm$  SEM, n=8 animals/group. Individual data points are coloured according to the 7 litters used.



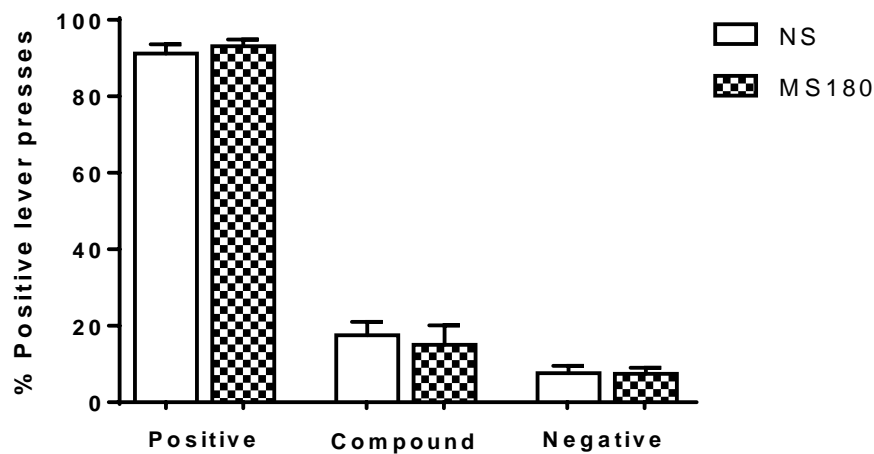
**Figure S3.** Maternal separation (MS180) animals showed no difference in approach latency in a novel environment compared to non-separated (NS) animals. Data represents the mean  $\pm$  SEM,  $n=8$ /group.



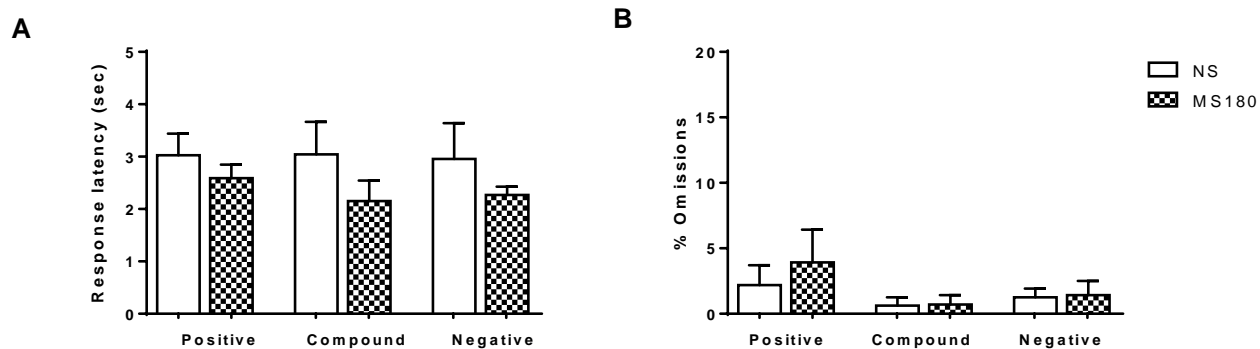
**Figure S4.** Maternal separation had no effect on total fluid consumption in the SPT. Data represents the mean  $\pm$  SEM,  $n=8$ /group.



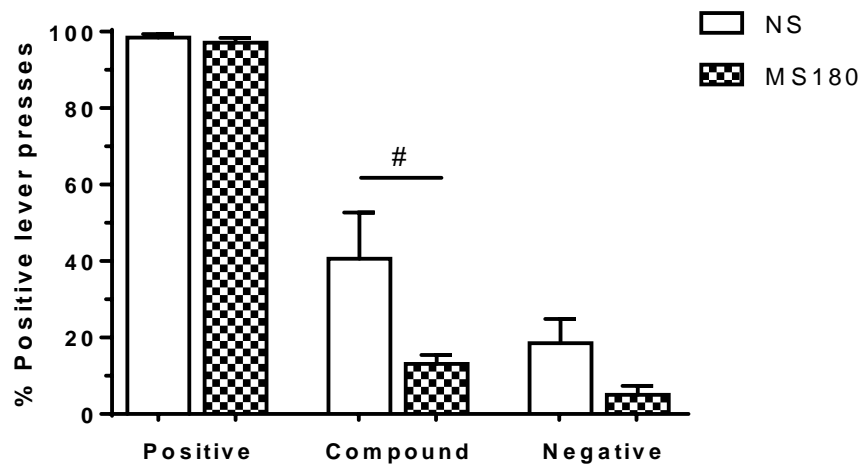
**Figure S5.** Maternal separation had no effect on animals' ability to discriminate the positive and negative cues in the low-reward JBT. There was no difference in responses to the compound cue. Data represents the mean  $\pm$  SEM percentage of lever presses on the positive lever,  $n=8$ /group.



**Figure S6.** Maternal separation had no effect on the response latency (A) or percentage of trial omissions (B) of animals in the low-reward JBT. Data represents the mean  $\pm$  SEM,  $n=8$ /group.



**Figure S7.** Maternal separation had no effect on animals' ability to discriminate the positive and negative cues in the high-reward JBT, however normal animals made more positive lever presses in response to the compound cue compared to the MS180 group. Data represents the mean  $\pm$  SEM percentage of lever responses on the positive lever, # $p < 0.05$ ,  $n = 8/\text{group}$ .



**Figure S8.** Maternal separation had no effect on the response latency (A) or percentage of trial omissions (B) of animals in the high-reward JBT. Data represents the mean  $\pm$  SEM,  $n = 8/\text{group}$ .

